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Unbalanced growth in mouse cells with amplified *dhfr* genes

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Abstract. When grown in the absence of methotrexate, cells carrying unstably amplified dihydrofolate reductase (*dhfr*) genes have a growth disadvantage that is a function of their level of gene amplification. Although this growth disadvantage is thought to drive the loss of unstably amplified *dhfr* genes in the absence of methotrexate, its mechanism is not understood. The present studies of murine cell lines with different levels of *dhfr* gene amplification demonstrate that such cells experience increased unbalanced growth (excess RNA and protein content relative to DNA content) with increased levels of *dhfr* gene amplification. Stathmokinetic analysis of a cell line with unstably amplified *dhfr* genes showed that the unbalanced growth was associated with a very low rate of G_1/S transit, which suggests that amplified DNA sequences may activate a cell cycle checkpoint at the G_1/S boundary. Hydroxyurea, which is known to induce rapid elimination of amplified genes at sub-cytotoxic concentrations, also inhibits the cell cycle at the G_1/S transition and causes unbalanced growth. Earlier work has shown that hydroxyurea selectively targets those cells within the heterogeneous drug resistant cell populations which have the highest amplified gene dosage. The finding that unstable gene amplification and hydroxyurea have similar effects on the cell suggests that hydroxyurea may achieve this selective targeting by pushing those cells with the highest levels of gene amplification over a critical stress threshold to cause growth arrest or cell death.

The high frequency of chromosomal rearrangement in tumours led to the idea that cancer cells are genetically unstable (Nowell 1976). Double minute chromosomes and homogeneously staining regions, which are now known to be karyotypic indicators of gene amplification, have long been recognized as chromosome aberrations unique to cancers (Cowell 1988). Gene amplification typically leads to elevated expression of the genes involved. Over-expression of oncogenes and drug resistance genes due to gene amplification contributes to tumour progression and drug resistance in cancer (Alioto & Schwab 1986, Stark 1986, Brison 1993).

When genes are unstably amplified, they are carried on episomes or double minute chromosomes. In both cases, they are acentric and randomly distributed between daughter cells at mitosis (Schimke et al. 1980, Kaufman, Brown & Schimke 1981, Cowell 1982, Barker

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1982). This leads to a cell population that is very heterogeneous with respect to amplified gene dosage. In the presence of a selection pressure (for instance, the drug methotrexate), those cells in the population with the highest levels of gene amplification (the *dhfr* gene in the case of methotrexate resistance) will have the highest levels of resistance to the drug. In the absence of a selection pressure, however, the growth advantage of cells with higher levels of genes amplification is reversed and the cells with the lowest level of gene amplification grow more rapidly (Kaufman *et al.* 1981). The growth advantage associated with lower levels of gene amplification is thought to be the basis for the progressive loss of unstably amplified genes from cells grown in the absence of the selecting drug. Uneven partitioning of the acentric double minutes or episomes at mitosis would lead to daughter cells with an even lower level of gene amplification and a still higher growth rate.

Hydroxyurea is an anticancer drug with a high therapeutic index, which suggests that it has significant selective effects against cancer cells. Our previous studies have demonstrated that sub-cytotoxic levels of hydroxyurea induce the rapid elimination of unstably amplified *dhfr* genes from methotrexate-resistant mouse cells (Snapka & Varshavsky 1983). Hydroxyurea has been shown to have the same effect on amplified oncogenes in human cancer cells (reviewed in Snapka 1992). Camptothecin, a topoisomerase I inhibitor, was also shown to be especially effective at eliminating amplified *dhfr* genes (Wani & Snapka 1990). In earlier studies we examined the relationship between amplified gene dosage and sensitivity to hydroxyurea and camptothecin (Wani, Strayer & Snapka 1990). By flow sorting a methotrexate-resistant cell population with an average *dhfr* gene dosage of 200 copies per cell into subpopulations with higher and lower levels of *dhfr* gene amplification, we were able to show that the cells with the highest *dhfr* gene dosage were much more sensitive to hydroxyurea and camptothecin. The goal of the present studies was to examine the basis for the sensitivity of cells with *dhfr* gene amplification to agents such as hydroxyurea. The finding that *dhfr* gene amplification is associated with unbalanced growth suggests that amplified DNA sequences, like hydroxyurea and other S-phase specific drugs, may cause unbalanced growth. This, in turn, suggests a mechanism by which drugs such as hydroxyurea can cause the rapid loss of amplified genes from drug-resistant cell populations.

MATERIALS AND METHODS

Determination of dhfr gene dosage

Cellular DNA was isolated and *dhfr* gene dosage was measured by slot blot hybridization as described (Wani & Snapka 1990). The measurement of *dhfr* gene amplification in methotrexate-resistant cell lines is relative to the *dhfr* gene dosage of methotrexate-sensitive parental lines which, by definition, have a *dhfr* gene dosage of 1X. The α-globin gene dosage is assumed not to vary with methotrexate-resistance and is used as an internal loading control in dot blot hybridization experiments.

Cell lines and selections for methotrexate resistance

3T6-derived methotrexate-resistant cells

3T6R.3 cells were subcloned from mouse 3T6 cells by one-step selection in 300 nM methotrexate (Snapka & Varshavsky 1983). To obtain cells with higher levels of *dhfr* gene amplification, 3T6R.3 cells were grown in progressively higher concentrations of methotrexate. The cells in this series are 3T6 (parental, methotrexate-sensitive, *dhfr* gene dosage 1X), 3T6R.3 (resistant to 300 nM methotrexate, *dhfr* gene dosage 7X), 3T6R.3-19

(resistant to 19 μM, *dhfr* gene dosage 13X), and 3T6R.3-80 (resistant to 80 μM methotrexate, *dhfr* gene dosage 16X).

CC1-derived methotrexate-resistant subclones
CC1 cells are a polyclonal population of NIH 3T3 cells carrying the retroviral vector pzIPNeoSV(X)1 without an expressed gene. The methotrexate-resistant subclones were obtained in single-step *in-vitro* selections for resistance to 100 nM methotrexate (Wani & Snapka 1989). The cells in this series are: CC1 (parental, polyclonal, methotrexate-sensitive, *dhfr* gene dosage 1X), CC1(1) a methotrexate-sensitive subclone of CC1 which shows much higher levels of DNA and RNA per cell, CC1R1 (*dhfr* gene dosage 10X), CC1R2 (*dhfr* gene dosage 12X), CC1R3 (*dhfr* gene dosage 14X), CC1R5 (*dhfr* gene dosage 10X), CC1R6 (*dhfr* gene dosage 10X).

Growth curves
Growth curves for each cell line were performed in the absence of methotrexate. Cells for growth curves were grown to 70% confluence, and then washed five times with methotrexate-free medium (20 ml) over a period of 48 h. This procedure is a more rigorous version of the washing procedure used to remove methotrexate from cells for uptake of fluorescein methotrexate (Wani & Snapka 1990). Specifically, cells were grown to 70% confluence in 100 mm plastic cell culture plates. The drug-containing medium was then removed, and the cells were rinsed with methotrexate-free medium. They were then covered with 20 ml of methotrexate-free medium for 4 h. This was followed by changes of methotrexate-free medium for 6 h, 12 h and finally for 26 h. The cells were then trypsinized, counted with a haemocytometer, and plated at 2×10^5 cells/plate to start the growth experiment. At the times indicated, duplicate plates were harvested by trypsinization and the number of cells per plate was determined by counting with a haemocytometer. The points for the growth curves were obtained by averaging the counts from three different duplicate plates.

Acridine orange multiparameter flow cytometry
An EPICS ELITE multiparameter flow cytometer (Coulter Corp., Miami, FL) was used in all flow cytometry studies. The listmode data was analysed with WinList software (Verity Software House, Inc., Topsham, MA). The acridine orange method for differential staining of DNA and RNA was used to discriminate different compartments of the cell cycle (Darzynkiewicz 1990) for studies of cell cycle kinetics and unbalanced growth. Unbalanced growth was measured as 'α-r', the ratio of RNA to total nucleic acid (RNA + DNA) as described (Traganos, Darzynkiewicz & Melamed 1982). Acridine orange (Polyscience Inc., Warrington, PA; Electro Pure, cat. #04539) was used. Freshly prepared human monocytes were used as an internal control. Monocytes were isolated immediately before each experiment, using lymphoprep (Nycomed Pharma, Oslo, Norway) as recommended by the manufacturer.

Stathmokinetic analysis
Stathmokinetic analysis was done using acridine orange multiparameter flow cytometry (Darzynkiewicz, Traganos & Kimmel 1986, Traganos & Kimmel 1990). The use of the metaphase arresting agent, vinblastine, prevents mitosis so that the cells accumulate at this stage of the cell cycle, and cannot re-enter the cell cycle as G_1 cells. Under these conditions, the rate of emptying of the G_1 compartment and the rate of accumulation in the $G_2 + M$ compartment can be measured. An estimate of S-phase transit time can also be obtained. For

the stathmokinetic study, 3T6R.3-19 cells were washed with methotrexate-free medium as described above for the growth curve experiments. Both 3T6 cells and 3T6R.3-19 were seeded at 2×10^5 cells per plate (10-cm plates). The stathmokinetic experiments were started when the cells were in early log phase (2.6×10^6 cells/plate for 3T6 and 5.2×10^6 cells/plate for 3T6R.3-19). The metaphase arresting agent, vinblastine (Sigma Chemical Co., St. Louis, MO), was dissolved in deionized water immediately before use. The final concentration of vinblastine in the cell culture medium was 50 μg/ml. The cell cycle distribution for the early log phase cells was determined by multiparameter flow cytometry with acridine orange staining of DNA and RNA as described above. Based on the RNA and DNA content of the cells, windows were set for the G_1, S and G_2/M compartments of the cell cycle. These windows were used throughout the experiment.

Direct measurement of cell diameter

Confluent cell monolayers were trypsinized at 37°C after two rinses with warm phosphate buffered saline. The cell suspension was gently pipetted to break up clumps, and applied to a haemocytometer. Photographs of the cells suspension were taken in the central area of the haemocytometer grid (30× magnification), and enlarged photographic prints were made. Cell diameters were measured in millimetres on the photographic enlargements, and their true diameters were obtained by converting millimetres to microns based on the measured spacing of the haemocytometer grid lines (50 μm spacing). The size distributions in the methotrexate-resistant lines were compared to the size distribution of the parental drug sensitive line.

RESULTS

3T6 series

In the absence of methotrexate, cells with higher levels of *dhfr* gene amplification were found to have the lowest growth rates and the lowest maximum cell densities (Figure 1a). The mid log phase (maximum) growth rate for each line was inversely related to the level of *dhfr* gene amplification (Figure 1b). The decrease in maximum growth rate with increasing *dhfr* gene dosage appears to be exponential. In all four lines, the plateau phase of the growth curve corresponded with confluence of the cell layer. The decreased cell density at confluence suggests that the cells with higher *dhfr* gene dosage are larger than those with lower *dhfr* gene dosage. To confirm this, the confluent cells were trypsinized and measured directly on photomicrographs which included an etched scale of known dimensions. Direct measurement of cell size distributions in these four lines confirm that the cells with higher *dhfr* gene dosage were larger when the cells were grown in the absence of methotrexate (Figure 2).

Increased cell size is a well known feature of unbalanced growth, an excess of RNA and protein content over DNA content during S-phase of the cell cycle (Ross 1981, Ross 1976, Cohen & Studzinski 1967). S-phase specific drugs such as hydroxyurea are well known for causing unbalanced growth (Snapka 1992, Traganos *et al.* 1982, Frankfurt 1981). The growth curves in Figure 1 are similar to the previously reported progressive changes in growth for cells growing in increasing concentrations of hydroxyurea (Snapka & Varshavsky 1983, Wani *et al.* 1990). To determine the relationship between *dhfr* gene dosage and unbalanced growth, multiparameter flow cytrometric technology in combination with the metachromatic dye acridine orange was used to analyse unbalanced growth. Acridine orange emits at red wavelengths when complexed to RNA, and at green wavelengths when complexed to DNA. This allows simultaneous measurement of RNA and DNA content of the cells (Darzynkie-

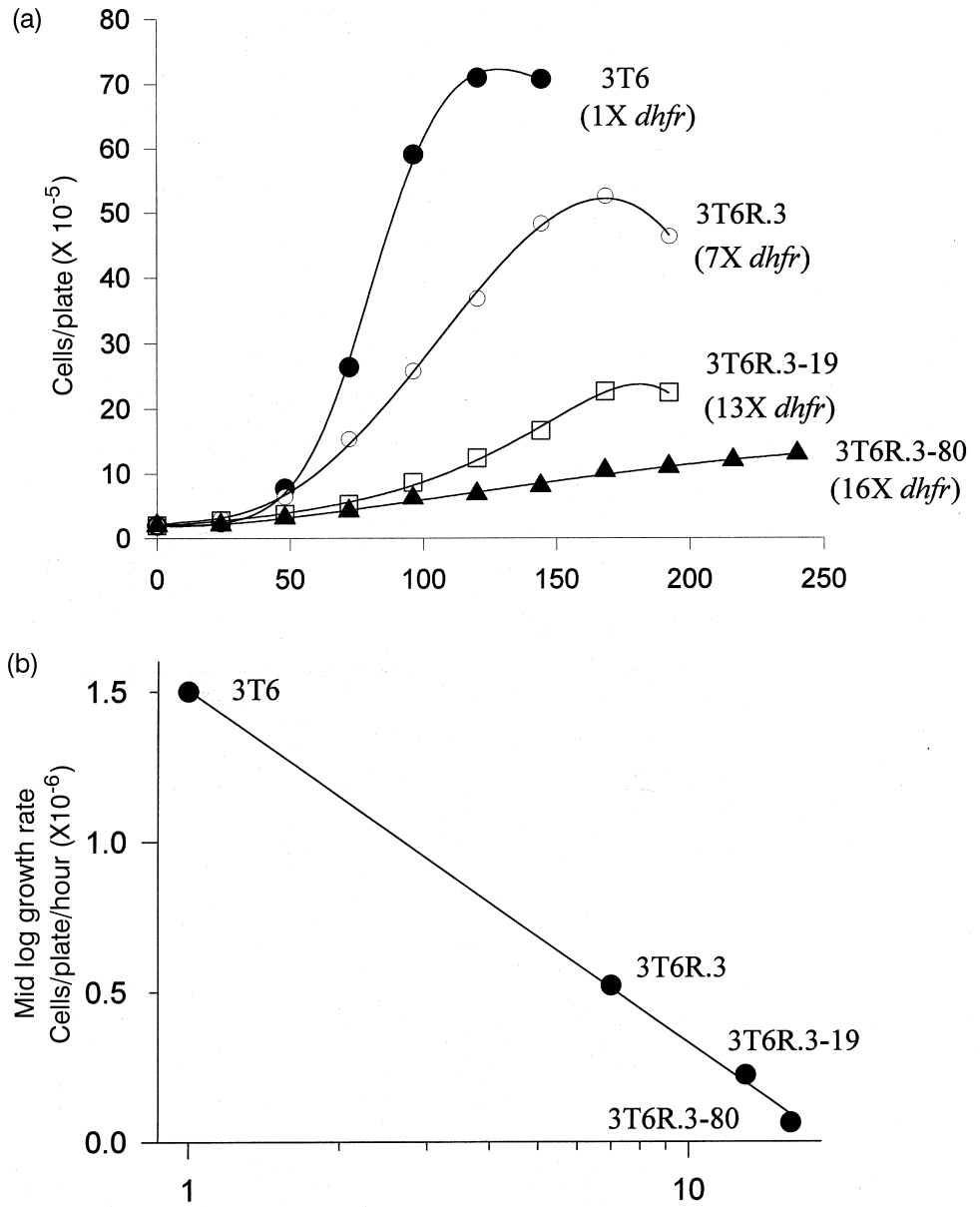

Figure 1. Growth of 3T6 cell lines as a function of *dhfr* gene amplification. (a) Growth curves. Cells were grown in the absence of methotrexate after five washes in methotrexate-free media over a 48 h period. 3T6 (parental methotrexate-sensitive 3T6 cells); 3T6R.3 (resistant to 300 nM methotrexate); 3T6R.3-19 (resistant to 19 μM methotrexate), 3T6R.3-80 (resistant to 80 μM methotrexate). The relative *dhfr* gene dosage for each cell line is indicated. (b) Relation between mid-log growth rate (derived from (a)) and *dhfr* gene copy number.

wicz 1990). The ratio of RNA to total nucleic acids (RNA + DNA), also known as the 'α-r value', has been shown to be a good measure of unbalanced growth (Traganos *et al.* 1982). The progressive shift to higher α-r values with increasing *dhfr* gene dosage shows that *dhfr* gene amplification is associated with unbalanced growth in these mouse fibroblasts (Figure 3).

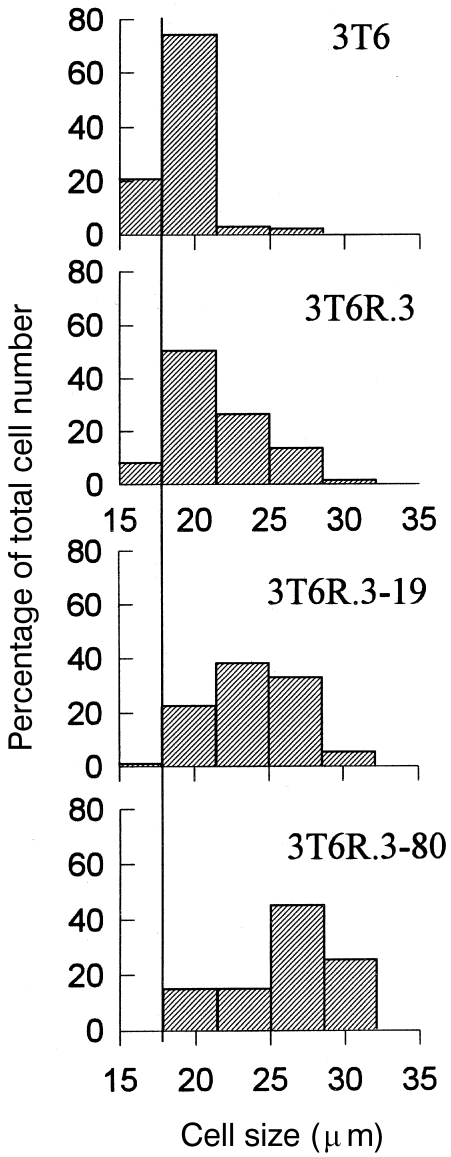

Figure 2. Direct measurement of cell size for methotrexate-resistant 3T6 cell lines. Cell suspensions were applied to a haemocytometer, photographs were taken at 30× magnification, and photographic enlargements were made. Cell diameters were measured (in millimetres) on the enlarged photographs, and were then converted to microns based on the spacing of the haemocytometer grid lines (50 μm grid spacing).

Forward laser light scatter is a well established indicator of cell size (Watson 1991). Right angle laser light scatter is usually associated with cell structure or granularity, but this parameter is also known to increase with cell size for cells of a given type (Seamer & Mandler 1992). Both forward and right angle light scatter increased with increasing *dhfr* gene dosage in this series (Figure 3). This is consistent with the direct measurements of cell size (Figure 2).

Stathmokinetic analysis of 3T6R.3-19 cells (relative *dhfr* copy number 13X) and the parental 3T6 cell line (Figure 4) demonstrates that the cells with amplified *dhfr* genes are very slow to exit G_1 compared to the parental cells. The slopes of the lines for G_1 exit indicate a mean G_1 duration of about 2 h for the parental 3T6 cells and a mean G_1 duration

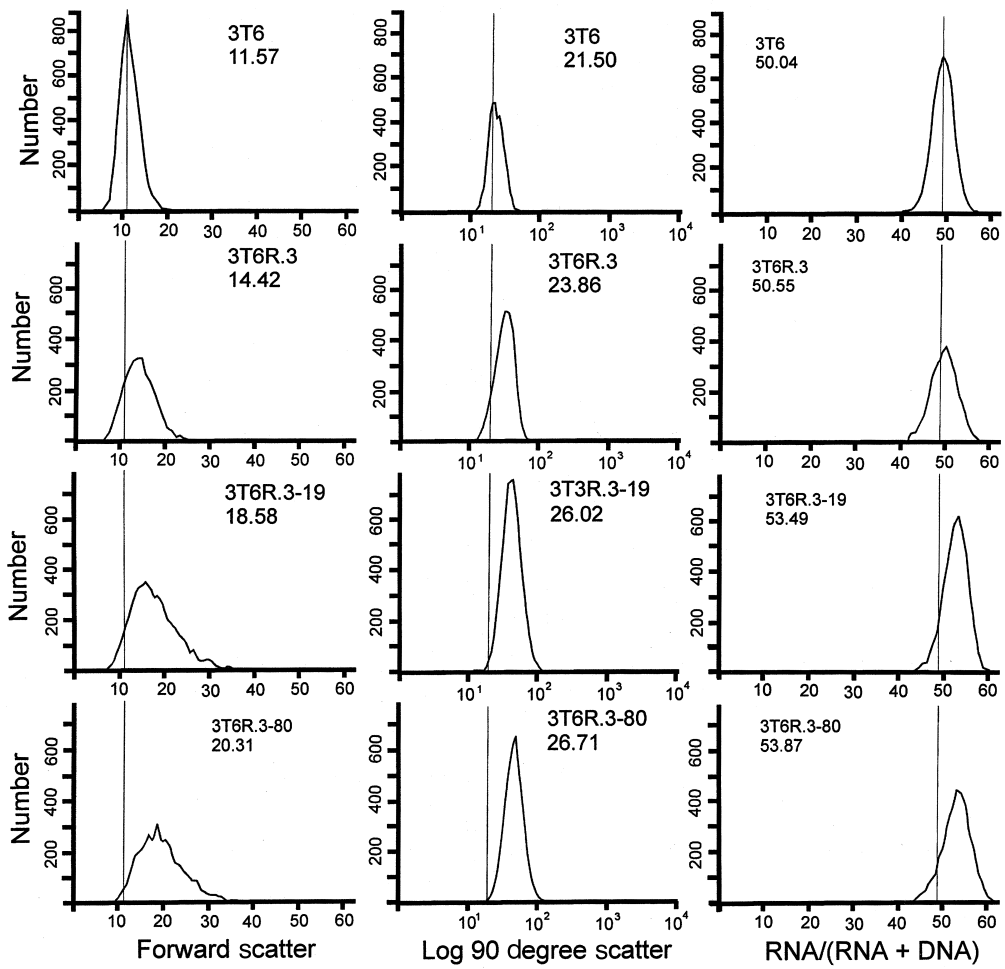

Figure 3. Flow cytometric analysis of unbalanced growth in methotrexate-resistant 3T6 cell lines. Horizontal rows represent the parental 3T6 cell line and the methotrexate-resistant sublines from Figure 1. Left column, forward light scatter; middle column, 90 degree light scatter; right column, α-r value (RNA/(RNA + DNA)). The peak value is indicated next to the cell line name in each graph. A vertical line is drawn through the value for the parental line in each column to help show the progressive shifts in values for the drug resistant sublines.

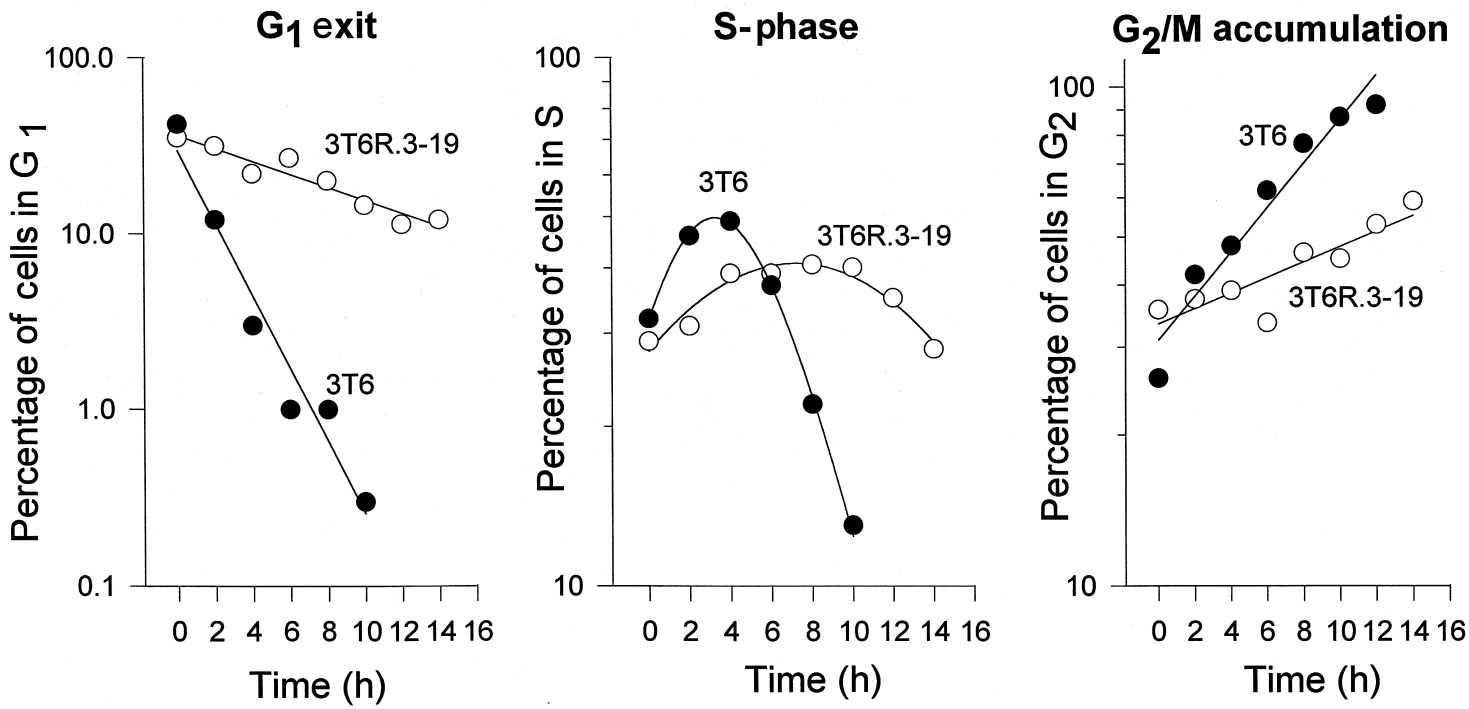

Figure 4. Stathmokinetic analysis of 3T6R.3-19 and parental 3T6 cell growth. With re-entry into the cell cycle as G_1 cells prevented by the metaphase blocking agent, it can be seen that the 3T6R.3-19 cells (*dhfr* copy number 13X) are very slow to exit G_1 relative to the methotrexate sensitive, parental 3T3 cells (left panel). The 3T6R.3-19 cells also had a very slow S-phase transit relative to the parental 3T6 cells (middle panel). The S-phase population of 3T6R.3-19 cells is still very high at a time when 3T6 cells have largely moved out of S-phase and into the G_2/M compartment. The accumulation of 3T6R.3-19 cells in the G_2/M compartment is also slow relative to the parental 3T6 line (right panel).

of about 12 h for the 3T6R.3-19 line (Traganos & Kimmel 1990). The 3T6R.3-19 cells are also slow to transit S-phase and slow to accumulate in G_2/M. It seems likely that the very long G_1 duration of 3T6R.3-19 cells may be rate limiting and thus responsible for much of the slow S-phase transit and the slow entry into the G_2/M compartment.

CC1 series

To confirm the relationship between unbalanced growth and gene amplification, another series of cell lines selected for methotrexate-resistance was studied. The parental CC1 cells and their methotrexate-resistant subclones have been previously described (Wani & Snapka 1989). All of the methotrexate-resistant CC1 subclones represent different single-step *dhfr* gene amplification events, all were selected for the same level of methotrexate resistance (100 nM) and all had about the same level of *dhfr* gene amplification (10–14X). The growth curves for the methotrexate-resistant subclones showed that they all grew to higher density than the polyclonal parental CC1 population (Figure 5). The extent of *dhfr* gene amplification had no effect on either the maximum cell density or the mid log growth rate. However, these differences could be due to less efficient contact inhibition in the subclones. Direct measurement of cell size in mid log phase cultures (Figure 6) indicated that the methotrexate resistant subclones were larger than the cells of the parental population when grown in the absence of methotrexate. Flow cytometric analysis of mid log phase cells grown in the absence of methotrexate indicated that the methotrexate-resistant subclones uniformly demonstrate unbalanced growth compared to the parental cell population (Figure 7). Analysis of forward and right angle laser light scatter characteristics also indicated that the average cell size was larger in the methotrexate-resistant clones with *dhfr* gene amplification.

DISCUSSION

The goal of this study was to discover the basis for the increased sensitivity of cells with amplified genes to S-phase specific drugs such as hydroxyurea. Earlier work (Wani *et al.* 1990) has suggested that this increased sensitivity may be the basis for the ability of these drugs to cause the rapid loss of amplified genes. A clonally derived series of mouse cell lines selected for increasing methotrexate resistance due to *dhfr* gene amplification showed reduced growth rates with increasing average *dhfr* gene copy number when grown in the absence of methotrexate. This result is in good agreement with the earlier findings of Kaufman *et al.* 1981. The reduced growth rate was associated with reduced maximum cell density. Hydroxyurea, a well known inducer of unbalanced growth, had been shown to have the same effect on cultured mouse cells in earlier studies on drug-induced loss of amplified genes (Snapka & Varshavsky 1983). Since cells in unbalanced growth tend to be enlarged, we speculated that the reduced maximum growth might be due to earlier contact inhibition of the larger cells. This in turn suggested unbalanced growth might be a function of the level of gene amplification. Increased cell size as a function of *dhfr* gene copy number was demonstrated by direct measurement of the cells, and was confirmed by analysis of forward and right angle laser light scattering data. Increased unbalanced growth with increased *dhfr* gene copy number was demonstrated directly by multiparameter flow cytometry with acridine orange.

To confirm the results with the 3T6-derived cell lines, we examined cell size and unbalanced growth in a series of mouse 3T3 fibroblast clones selected for methotrexate resistance due to *dhfr* gene amplification (CC1 series). In contrast to the 3T6 series in which

a single clonal isolate was adapted to growth in progressively higher methotrexate concentrations, the CC1 lines were all independently derived clones obtained in single-step selections for methotrexate resistance. Although the methotrexate-resistant CC1 clones did not show reduced growth rates and reduced maximum cell densities relative to the polyclonal CC1 population, direct measurements of cell size and flow cytometry measurements of laser light scatter both indicated that the cells in these clonal lines were larger on average than the cells of the parental population. Acridine orange flow cytometry showed that each of the clones with *dhfr* gene amplification was in unbalanced growth when grown in the absence of methotrexate. The results with the methotrexate-resistant CC1 clones support the results of the study with the 3T6 series of lines selected for progressively higher methotrexate resistance. The fact that the CC1 clones with *dhfr* gene amplification did not show the same reduced growth rate and maximum cell density relative to the parental CC1 population may

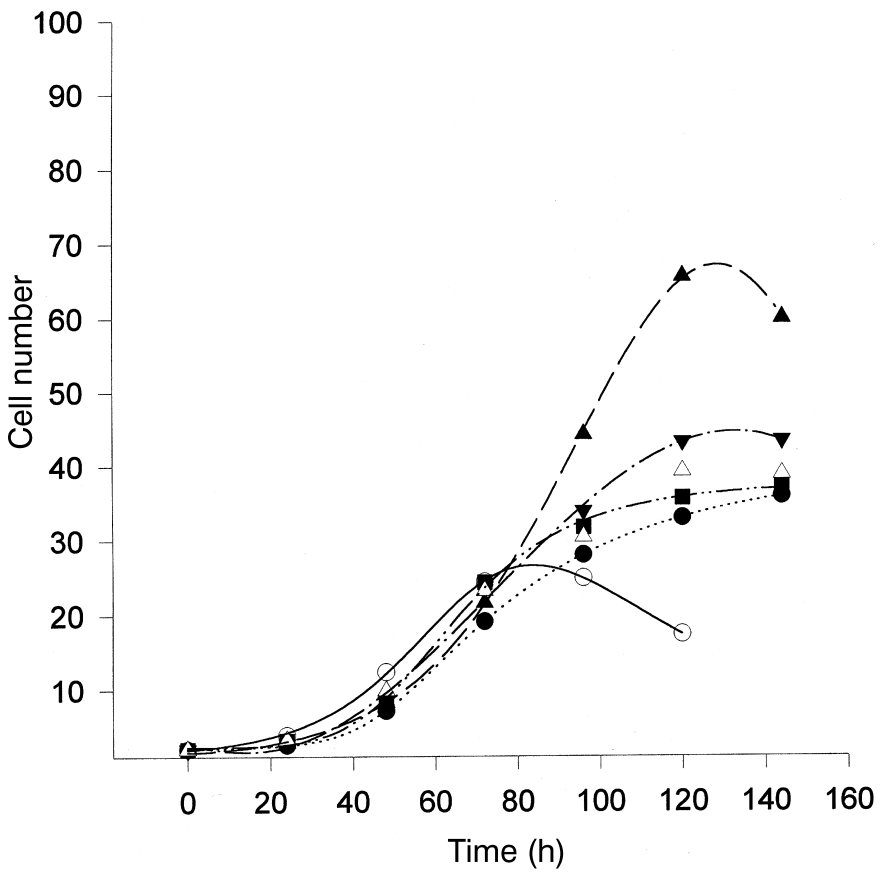

Figure 5. Growth curves for the parental CC1 population and methotrexate-resistant CC1 cell lines. The parental CC1 population is derived from a pool of NIH 3T3 cells carrying the retroviral vector pZIPNeoSV(X)1 without an expressed gene. The methotrexate-resistant subclones were obtained in single step *in vitro* selections for resistance to 100 nM methotrexate. CC1(1) a methotrexate-sensitive subclone derived from the CC1 population by plating at high dilution. It has much higher levels of DNA and RNA per cell than cells of the mixed CC1 population. Clones selected for resistance to 100 nM methotrexate: CC1R1 (*dhfr* gene dosage 10X), CC1R2 (*dhfr* gene dosage 12X), CC1R3 (*dhfr* gene dosage 14X), CC1R5 (*dhfr* gene dosage 10X), CC1R6 (*dhfr* gene dosage 10X). ○, CC1; ●, CC1R1; ▼, CC1R2; ■, CC1R3; ▲, CC1R5; △, CC1R6.

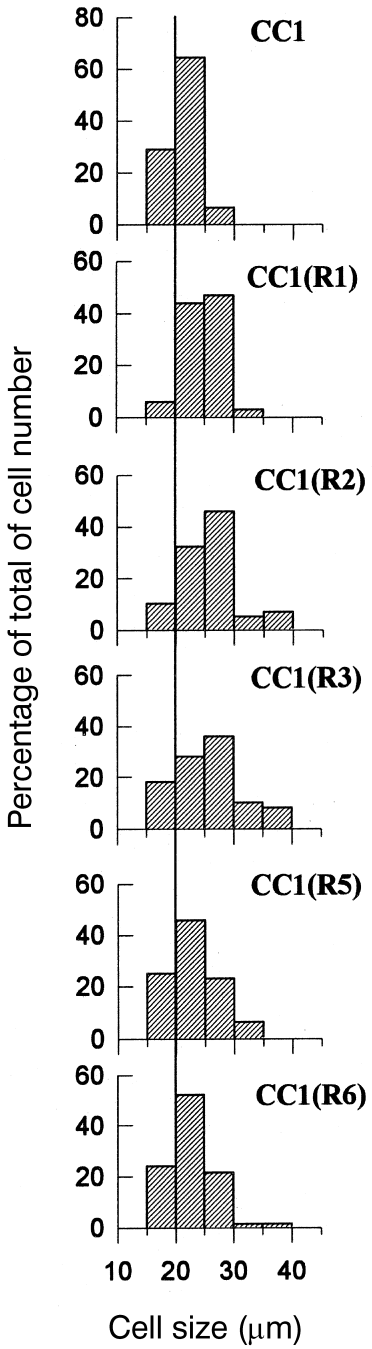

Figure 6. Direct measurement of cell size for methotrexate-resistant CC1 cell lines. Cell diameters were measured as described in Figure 2.

be due either to reduced contact inhibition in the CC1 cells generally or to the clonal nature of the drug resistant lines and the polyclonal nature of the parental population.

These results confirm the inverse relationship between the level of *dhfr* gene amplification and the growth rate of cells in the absence of methotrexate, and show that cells with episomally amplified genes experience unbalanced growth. The results of the 3T6 clone

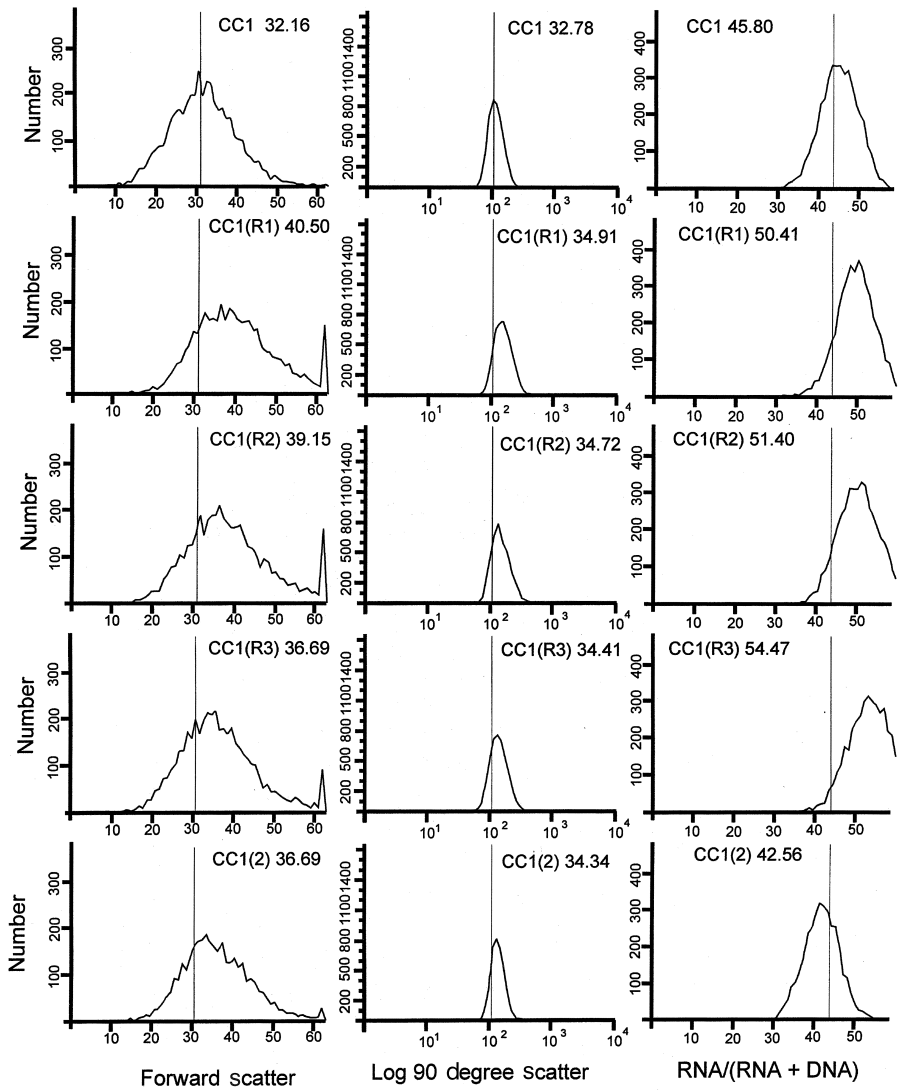

Figure 7. Light scatter and unbalanced growth in methotrexate-resistant CC1 cell lines. The parental methotrexate sensitive CC1 population, one methotrexate sensitive subclone, CC1(1), and the methotrexate resistant subclones (see Figure 5 for *dhfr* gene dosages) are represented in the horizontal rows. Left column, forward light scatter; middle column, 90 degree light scatter; right column, α-r value (RNA(RNA + DNA)). The peak values are indicated next to the cell line name in each graph. Vertical lines are drawn through the peak of the parental CC1 population in each case to help visualize the shifts in values for the subclones.

selected for higher levels of methotrexate resistance showed increased unbalanced growth with increased levels of gene amplification. Unstably amplified genes, in other words, appear to have effects on cells similar to those of S-phase specific drugs such as hydroxyurea. These results suggest a mechanism for drug-induced loss of amplified genes. Although slot blot analysis measures an average gene dosage for a cell population, cell lines with unstably amplified genes are very heterogeneous with respect to the amplified gene dosage in individual cells (Kaufman *et al.* 1981, Snapka 1992). Such drug-resistant cell populations typically contain small numbers of cells with many times the population average gene dosage. Those cells within the population with the highest levels of gene amplification would be experiencing the most severe unbalanced growth. Additional unbalanced growth induced by low concentrations of an S-phase agent like hydroxyurea could push these cells over a critical stress threshold, resulting in growth arrest or cell death. Selective growth inhibition or killing of those cells with above average amplified gene dosage would greatly accelerate the drop in the average gene dosage within the cell population.

There is evidence that the presence of amplified genes slows the growth of cells even in the presence of the selecting drug (Haber & Schimke 1981, Shen, Pastan & Gottesman 1988). Consistent with this, hydroxyurea and camptothecin can selectively target cells with higher levels of *dhfr* gene amplification even in the presence of the selecting drug, methotrexate (Wani *et al.* 1990). This is important since the selection pressure for oncogene amplification cannot be removed in the same way that a cytotoxic drug can be removed. Because human oncogenes are commonly over-expressed due to unstable gene amplification, and since unstably amplified human oncogenes are also subject to drug-induced loss (Von Hoff *et al.* 1992, Von Hoff *et al.* 1991), it is possible that selective targeting of the cells with higher levels of gene amplification contributes significantly to the favourable therapeutic index of drugs such as hydroxyurea.

Stathmokinetic analysis of 3T6R.3-19 cells showed that the unbalanced growth is associated with a greatly reduced rate of G_1/S transit. Our studies do not indicate whether the inhibition happens at the restriction point, which normally occurs about two-thirds of the way through G_1, or at the G_1/S boundary. Growth arrest at the restriction point can be caused by DNA damage or metabolite imbalance, and can be either p53 dependent or p53 independent (Dou, An & Will 1995, Chen *et al.* 1996, Linke *et al.* 1996, Kaufmann & Paules 1996). Additional DNA damage-sensitive cell cycle checkpoints operating after the restriction point, in late G_1, the G_1/S boundary or in S-phase have also been reported (Kaufmann & Paules 1996, Lee, Larner & Hamlin 1997). Inhibition of G_1/S transit could occur by a cell cycle checkpoint mechanism if acentric, episomal DNA were sensed by cells as a form of DNA damage. Hydroxyurea is thought to act after the restriction point at the G_1/S boundary or in very early S-phase (Frankfurt 1981, Traganos *et al.* 1982, DAnna, Church & Tobey 1986). Amplified DNA sequences could also inhibit the cell cycle at the G_1/S boundary or early S-phase if they compete with other replicons for critical proteins required for initiation of DNA replication.

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